Supplemental Text

Materials and Methods

Northern analysis

Poly(A) RNA was isolated from yeast transformed with the CrPV, IAPV or TSV IGR IRES dual luciferase reporter using the oligotex kit (Qiagen). The RNA was separated on a denaturing agarose gel (0.8% agarose, 16% formaldehyde) in MOPS buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA at pH7.0) and transferred to a zeta-probe membrane (Bio-Rad). The α^{32}P-dCTP (PerkinElmer) radiolabeled firefly luciferase probe was generated using the prime-a-gene kit (Promega) and the SphI-AvaI fragment of the firefly luciferase gene (bases 403-1062) served as the template.

Results

Monocistronic transcripts are generated from the dicistronic reporter

The northern analysis demonstrates that in yeast cells transformed with the dual luciferase reporter that, in addition to the full length dicistronic message, a transcript is present that contains firefly luciferase that is the approximate size for a monocistronic transcript. This suggests that a functional firefly luciferase could be synthesized from the monocistronic message if there is an in frame upstream start codon contained within the IGR IRES. Because the monocistronic transcript is observed in all dicistronic reporters regardless of the IRES inserted we suspect that the cryptic promoter lies within the *Renilla* coding region.

PKIII mutant

In the crystal structure of the PSIV IGR IRES, SL2.3 bends upward and juxtaposes SL2.1 (1). Based on the Cryo-EM data of the ribosome bound to the IGR IRES, these terminal loop regions are predicted to interact directly with the 40S subunit (2,3). Mutagenesis data are consistent with the structural data and suggest that SL2.1 and SL2.3 are necessary for recruiting the 40S ribosome (4,5). Therefore, if these loops are not properly positioned the IGR IRES may be unable to bind to the ribosome. The published IAPV sequence has the propensity to form an extended PKIII which could lock SL2.3 into the down position (Figure S2A, dashed lines). To test if this is partially responsible for the decreased IRES activity of IAPV in yeast, a linker mutant uc6486aa, was created that prohibits interaction between the stem of SL2.3 and the linker region upstream of PKIII (Figure S2A, upper gray box). This mutant did not increase activity of the wild-type IAPV IGR IRES or the more active $IAPV_{gcu}$ in yeast suggesting that the proposed extension of PKIII did not affect IRES activity (Figure S2B). Mfold calculations of the IAPV IRES routinely paired the bases of PKIII but did not predict an extension outward suggesting that it is unlikely an extension of PKIII was occurring *in vivo* even with available base-pairing interactions (6). These data suggest that the most likely difference between the IAPV IGR IRES and the closely related ABPV and KBV IRESs is due to the

sequence of the first codon of ORF2 rather than structural disturbances in the IRES.

Supplementary Figure Legends

Figure S1: Northern analysis demonstrates IGR IRES plasmids have cryptic promoter activity in yeast. **(A)** Short exposure allows visualization of the dicistronic message. **(B)** Overexposure of the same blot allows visualization of small monocistronic bands. RiboRuler (Fermentas) molecular weight markers are indicated (left) and the transcripts based on size of the dicistronic and firefly luciferase monocistronic messages are indicated to the right of the blots.

Figure S2: The sequence in the linker region between the first stem and PKIII does not affect IAPV IGR IRES activity. **(A)** A diagram of the IAPV $_{uc}6486_{aa}$ mutation that was engineered into either the wild-type IAPV IGR IRES or the more active $IAPV_{gcu}$ to prohibit extension of PKIII. **(B)** Translational activity of the mutants were assessed in wild-type yeast and normalized to the control constructs without the linker mutation.

Figure S3: Temperature differences do not affect *Renilla* luciferase values in yeast. The *Renilla* luciferase values from all IGR IRES dicistronic reporters in Figure 6A. Error bars represent SE for n=11.

References

1. Pfingsten, J.S., Costantino, D.A. and Kieft, J.S. (2006) Structural basis for ribosome recruitment and manipulation by a viral IRES RNA. *Science*, 314, 1450-1454.
2. Spahn, C.M., Jan, E., Mulder, A., Grassucci, R.A., Sarnow, P. and Frank, J. (2004) Cryo-EM visualization of a viral internal ribosome entry site bound to human ribosomes: the IRES functions as an RNA-based translation factor. *Cell*, 118, 465-475.
3. Schuler, M., Connell, S.R., Lescoute, A., Giesebrecht, J., Dabrowski, M., Schroeer, B., Mielke, T., Penczek, P.A., Westhof, E. and Spahn, C.M. (2006) Structure of the ribosome-bound cricket paralysis virus IRES RNA. *Nat Struct Mol Biol*, 13, 1092-1096.
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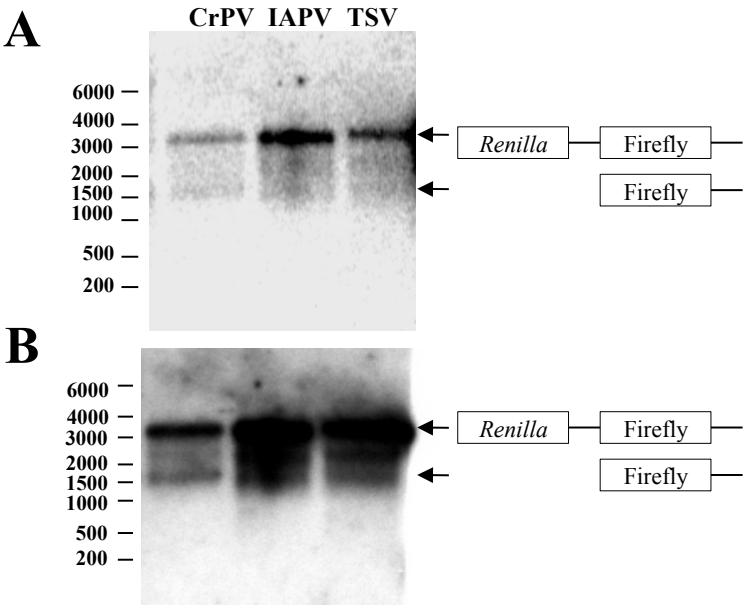
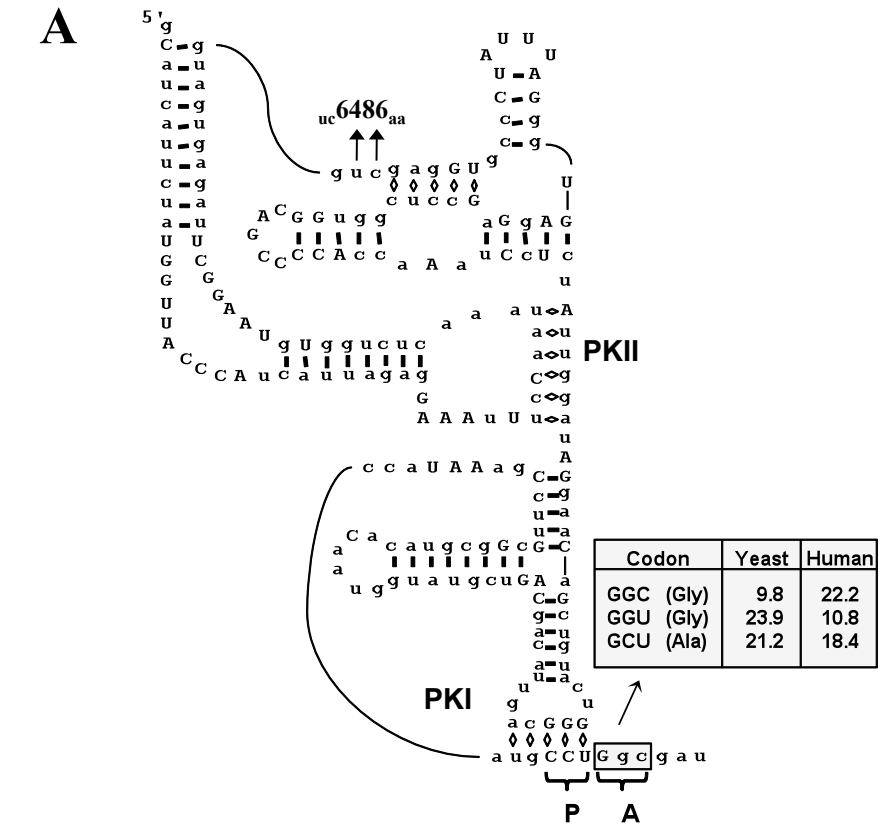
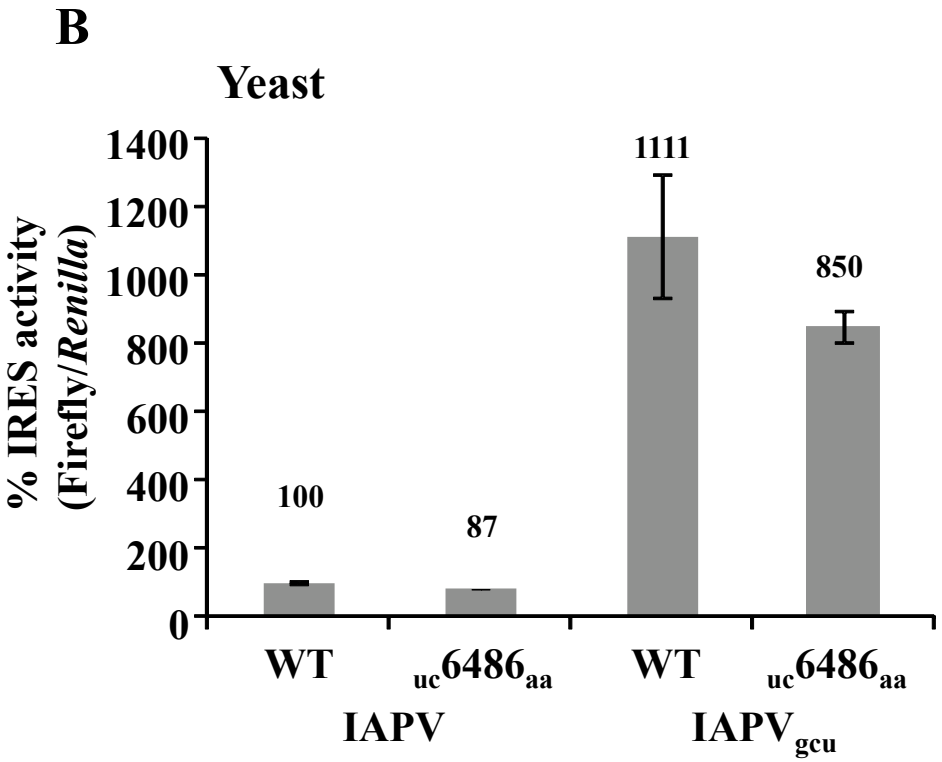
5. Costantino, D. and Kieft, J.S. (2005) A preformed compact ribosome-binding domain in the cricket paralysis-like virus IRES RNAs. *RNA*, 11, 332-343.
6. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, 31, 3406-3415.

S1- supplementary figure 1

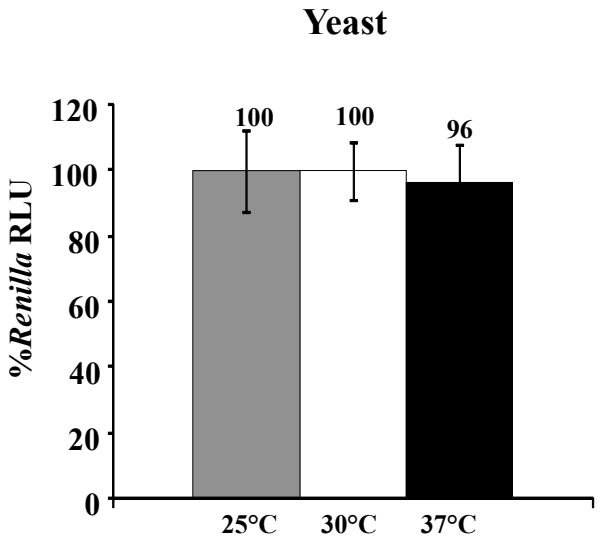


S2- supplementary figure 2





S2- supplementary figure 2



Primer Set	#	Sense	Antisense
Insert SacII	P1	CTACATTTCAAGATACCGCGGAAGACGCCAAAAACAT	ATGTTTTTGGCGTCTTCCGCGGTATCTTGAAATGTAG
HiPV	P2	ctcgagTGAGAGTTTCAAACATTGTGC	ccgcggAATTATTATTATTTGCTAGTG
HoCV-1	P3	ctcgagGACGAGGACTAAGTGTGAAC	ccgcggTTTGTTGTGATGTTGCGAGTCTGG
PSIV	P4	ctcgagCGACACGCGGCCTTCCAAGC	ccgcggAAAATTCTTTTTCTTGAAGTG
ABPV	P5	ctcgagGTAATTTGGGAATCGCAACAC	ccgcggTTGTTTCTTGATCGGCAGGTATG
IAPV	P6	ctcgagGTATAGTGTTCTGGAGGC	ccgcggTTGAATCGCCAGGCATGGTATTTCG
KBV	P7	ctcgagATATAATACTTGAGATGCACTGTTTCATGGTTACCC	ccgcggTTTCTTGGTTATCAGCAGGTATGG
KBV flank	P8	GGATATGCTCAGGAGCGGTATC	CCTTCTCAATGGCATTCTCCGC
SINV-1	P9	ctcgagAACGTTTCTCTTCTGG	ccgcggTATTGTTTGATTGAG
TSV	P10	ctcgagTAGCACCACCCGATCGTAAACTCC	ccgcggTTTCAACTGGGTTAGCAGGC
CrPV ΔSacII	P11	CATTTCAAGATACCGAAGACGCCAAAAAC	CTTTATGTTTTTGGCGTCTTCGGTATCTTGAAATGTAGCAG
HiPV ΔSacII	P12	AATTTCACTAGCAAATAATAATAATTCCGAAGACGCCAAAAACATAAAGAAAG	CTTTCTTTATGTTTTTGGCGTCTTCGGAATTATTATTATTTGCTAGTGAAATT
HoCV1 ΔSacII	P13	CAACATCACAACAAACCGAAGACGCCAAAAAC	GTTTTTGGCGTCTTCGGTTTGTTGTGATGTTGCG
PSIV ΔSacII	P14	TCTCACTTCAAGAAAAAGAATTTACCGAAGACGCCAAAAACATAAA	TTTATGTTTTTGGCGTCTTCGGTAAATTCTTTTTCTTGAAGTGAGA
ABPV ΔSacII	P15	GCCGATCAAGAAACAACCGAAGACGCCAAAAACATA	TATGTTTTTGGCGTCTTCGGTTGTTTCTTGATCGGC
IAPV ΔSacII	P16	GGCGATTCACAACAAACCGAAGACGCCAAAAAC	CTTTATGTTTTTGGCGTCTTCGGTTTGTTGTGAATCGCC
KBV ΔSacII	P17	GATAACCAAGAAACCGAAGACGCCAAAAACA	GTTTTTGGCGTCTTCGGTTTCTTGGTTATC
SINV1 ΔSacII	P18	CCTGCTCAATCAAACAATACCGAAGACGCCAAAAACATA	TATGTTTTTGGCGTCTTCGGTATTGTTTGATTGAGCAGG
TSV ΔSacII	P19	CTAACCCAGTTGAAACGGAAGACGCCAAAAAC	GTTTTTGGCGTCTTCCGTTTCAACTGGGTTAG
IAPV_GCU	P20	GAAATACCATGCCTGCTGATTCACAACAAACC	GGTTTGTTGTGAATCAGCAGGCATGGTATTTC
KBV_GGC	P21	GTTTCGAAATACCATACCTGGCGATAACCAAGAAACCGAAG	CTTCGGTTTCTTGGTTATCGCCAGGTATGGTATTTCGAAAC
IAPV_GGU	P22	GAAATACCATGCCTGGTGATTCACAACAAACC	GGTTTGTTGTGAATCACCAGGCATGGTATTTC
IAPV 6486aa	P23	GTAAGGCTTAGAGTGATGGAAGAGGTGCCCTATTTAGGG	CCCTAAATAGGGCACCTCTTCCATCACTCTAAGCCTTAC
Fluc Rev	P24		CAGCGTAAGTGATGTCCACCT
PKI analysis			
HoCV1 cu-ga	P25	CATCATTAGAGACCAGAGACGCAACATCACAACAAACC	GGTTTGTTGTGATGTTGCGTCTCTGGTCTCTAATGATG
HoCV1 ag-uc	P26	GTGGACTTGGTGAGGATTGTCTTGACCTCATCATTAGAGA	TCTCTAATGATGAGGTCAAGACAATCCTCACCAAGTCCAC
KBV cc-gg	P27	ACATATTCGGCGTTTCGAAATACCATAGGTGCTGATAACCAAG	CTTGGTTATCAGCACCTATGGTATTTCGAAACGCCGAATATGT
KBV gg-cc	P28	GATAGAAACCGCTATATCGCCTAGCTATAGCAGTCGGATA	TATCCGACTGCTATAGCTAGGCGATATAGCGGTTTCTATC
TSV cc-gg	P29	CGTGGTGGGACTTAATTAATGGGTGCTAACCCAGTTGAAAC	GTTTCAACTGGGTTAGCACCCATTAATTAAGTCCCACCACG
TSV gg-cc	P30	AGTCCCAGTCACTTTGCCCAAAGTAGACAGCCGC	GCGGCTGTCTACTTTGGGCAAAGTGACTGGGACT
TSV aug-uga	P31	GGTGGGACTTAATTATGACCTGCTAACCCAGTTG	CTGGGTTAGCAGGTCATAATTAAGTCCCACC
TSV ca-uc	P32	TCCCAGTCACTTTGGGTCAAGTAGACAGCCGC	GCGGCTGTCTACTTGACCCAAAGTGACTGGGA
HiPV cu-ga	P33	AGGCTAAAGAATTTCAGAAGCAAATAATAATAATTC	GAATTATTATTATTTGCTTCTGAAATTCTTTAGCC
HiPV ag-uc	P34	CCTAGGTGCAGCCTTGTAGTTTTTCTGGACTTTAGGCTAA	TTAGCCTAAAGTCCAGAAAAACTACAAGGCTGCACCTAGG
PSIV cu-ga	P35	GCGAAAAGAATCTCAGATCAAGAAAAAGAATTTAC	GTAAATTCTTTTTCTTGATCTGAGATTCTTTTCGC
PSIV ag-uc	P36	CTCGCTCAAACATTATCTGGTGTTGTGCGAAAAG	TTCGCACAACACCAGATAATGTTTGAGCGAGCAC
KBV cc-gg	P37	ACATATTCGGCGTTTCGAAATACCATAGGTGCTGATAACCAAG	CTTGGTTATCAGCACCTATGGTATTTCGAAACGCCGAATATGT
KBV gg-cc	P38	GATAGAAACCGCTATATCGCCTAGCTATAGCAGTCGGATA	TATCCGACTGCTATAGCTAGGCGATATAGCGGTTTCTATC
IAPV cc-gg	P39	GCGTTCCGAAATACCATGGGTGGCGATTCACAACAAAC	GTTTGTTGTGAATCGCCACCCATGGTATTTCGGAACGC
IAPV gg-cc	P40	GATAGGAACAGCTGTACTGCCCAGTTACAGCAGTCGTATG	CATACGACTGCTGTAACTGGGCAGTACAGCTGTTCCTATC
IAPV aug-uga	P41	GGCGTTCCGAAATACCtgaCCTGGCGATTCACAAC	GTTGTGAATCGCCAGGtcaGGTATTTCGGAACGCC
IAPV ca-uc	P42	GAACAGCTGTACTGGGtcGTTACAGCAGTCGTATG	CATACGACTGCTGTAAcgACCCAGTACAGCTGTTC
ABPV cc-gg	P43	GAATGCAGCGTTCCGAAATACCATAGGTGCCGATCAAGAA	TTCTTGATCGGCACCTATGGTATTTCGGAACGCTGCATTC
ABPV gg-cc	P44	GGATAGGAACAGCTATATTGCCTAGTTGTAGCAGTTGTATTC	GAATACAACTGCTACAACTAGGCAATATAGCTGTTCCTATCC
SINV cc-gg	P45	GTTCCGAAATACCCAAAGGTGCTCAATCAAACAATAC	GTATTGTTTGATTGAGCACCTTTGGGTATTTCGGAAC
SINV gg-cc	P46	GACTAGGAACAGCTATATCGccTTGCTATAGCAGTCAGG	CCTGACTGCTATAGCAAggCGATATAGCTGTTCCTAGTC

Table S1